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Patterns of molecular genetic variation among African elephant populations

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Abstract

The highly threatened African elephants have recently been subdivided into two species, *Loxodonta africana* (savannah or bush elephant) and *L. cyclotis* (forest elephant) based on morphological and molecular studies. A molecular genetic assessment of 16 microsatellite loci across 20 populations (189 individuals) affirms species level genetic differentiation and provides robust genotypic assessment of species affiliation. Savannah elephant populations show modest levels of phylogeographic subdivision based on composite microsatellite genotype, an indication of recent population isolation and restricted gene flow between locales. The savannah elephants show significantly lower genetic diversity than forest elephants, probably reflecting a founder effect in the recent history of the savannah species.

Keywords: African elephant, microsatellites, phylogeography

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Introduction

African elephants have been the object of intense conservation study and debate in recent years, particularly as their numbers have decreased by nearly 50% in the 1980s (Cobb 1989). Although African elephants have been listed as endangered and protected since 1989, illegal poaching and habitat destruction continue to diminish and isolate remaining populations that are dispersed widely over 37 sub-Saharan African countries (Said *et al*. 1995; Barnes *et al*. 1999). Conservation management of African elephant populations is complicated by the presence of two distinct morphological forms known commonly as forest and savannah (or bush) elephants. Forest elephants occur throughout Africa's dense equatorial rain forests (White 1983), while savannah elephants are distributed across open savannahs and other habitats to the north, east and south of the forest elephants' range (Grubb *et al*. 2000). Thought commonly to be two subspecies, forest elephants exhibit distinct morphological characteristics relative to savannah elephants, including smaller rounded ears, a more compact body build and downward-pointing tusks (Matschie 1900; Grubb *et al*. 2000) that have elicited several proposals to reclassify forest elephants as a separate species (Frade 1931, 1955; Allen 1937; Azzaroli 1966; Tangley 1997; Grubb *et al*. 2000). Recent molecular genetic analyses based on regions of four nuclear genes (Roca *et al*. 2001) and a portion of the mitochondrial *cytochrome b* gene (Barriel *et al*. 1999) provide independent support for the recognition of two separate species of African elephant: *Loxodonta africana* (savannah or bush elephant) and *L. cyclotis* (forest elephant).

In this analysis we designate forest and savannah elephants as separate species (Grubb *et al*. 2000; Macdonald 2001; Roca *et al*. 2001) and test the extent and character of molecular genetic variation in three forest and 17 savannah elephant populations distributed across Africa. We examined allelic variation at 16 elephant-specific polymorphic microsatellite (also called short tandem repeat) loci and compared variation between species, broad geographical regions and specific geographical locales to ascertain the degree of genetic subdivision among African elephants. Our findings indicate considerable differentiation between African species, moderate differentiation between broad geographical regions and little differentiation between

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adjacent populations within species. One population, in Garamba National Park in the Democratic Republic of Congo, shows evidence of limited hybridization between forest and savannah elephants, consistent with a similar observation using nuclear genes (Roca *et al*. 2001).

Materials and methods

Samples

Samples were collected from African elephants in 20 locations (Fig. 1) primarily by biopsy darting (Georgiadis *et al*. 1994). Skin biopsies were collected as described in Karesh *et al*. (1989), stored in ethanol or frozen and imported to the United States in full compliance with specific Federal Fish and Wildlife Permits (endangered/threatened species and CITES Permits US 750138 and US756611). Samples were selected from a single individual within each group encountered and were collected from various regions of each geographical location. Based on morphological criteria and confirmation by nuclear gene phylogenetic analysis (Roca *et al*. 2001), animals from 17 sampling locations were characterized as savannah, while animals from three locations (Garamba, Lope and Dzanga Sangha) were classified as forest elephants. Garamba includes forest elephants (Groves & Grubb 2000), but has been a location where evidence of forest–savannah hybridization has been reported (Backhaus 1958; Roca *et al*. 2001). Samples were also collected and DNA isolated from 14 unrelated, captive Asian elephants in US zoos as described previously (Comstock *et al*. 2000). For most tissue samples, DNA was isolated using a commercially available kit (Qiagen, Valencia, CA, USA).

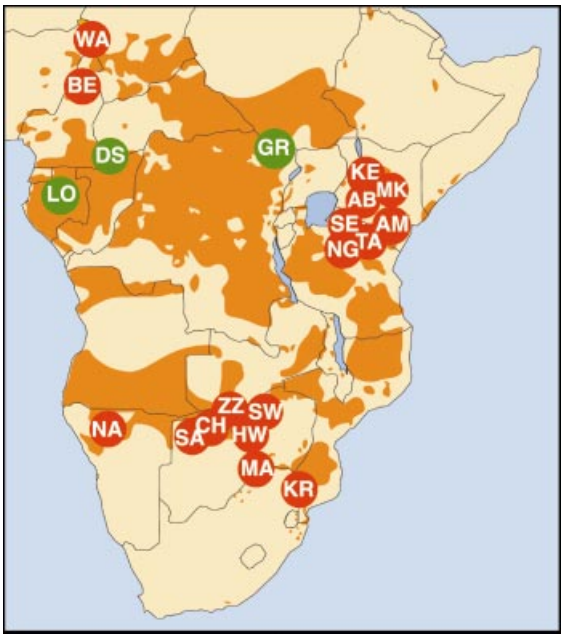

Fig. 1 Map of sub-Saharan Africa showing the collection sites. Orange indicates current elephant range (Barnes *et al*. 1999); historic range is in light brown (Grubb *et al*. 2000). Collection sites for savannah (red) and forest (green) elephants are shown. Population abbreviations: AB (Aberdares), AM (Amboseli), BE (Benoue), CH (Chobe), DS (Dzanga Sangha), GR (Garamba), HW (Hwange), KE (central Kenya), KR (Kruger), LO (Lope), MA (Mashatu), MK (Mount Kenya), NA (Namibia), NG (Ngorongoro), SA (Savuti), SE (Serengeti), SW (Sengwa), TA (Tarangire), WA (Waza), ZZ (Zambezi).

Microsatellite locus amplification and genotyping

The elephant samples were analysed using 16 polymorphic microsatellite loci containing CA dinucleotide repeats. Thirteen of these loci had been described previously by us and others (Nyakaana & Arctander 1998; Comstock *et al*. 2000). Three additional loci, FH126 (GenBank accession no. AF364124), FH127 (no. AF364125) and FH153 (no. AF364123) were isolated for this analysis.

Polymerase chain reaction (PCR) amplification of all loci was performed as described previously (Comstock *et al*. 2000) except for FH126, FH127 and FH153, which were amplified with the following primers: FH126F (5′-TCTGATAGGCTGGTGTAAGCTG-3′) and FH126R (5′-TCTCTCCTCCCTTCCCTCTC-3′), FH127F (5′-ACTGACCGGGAAGAGGAAGT-3′) and FH127R (5′-AGGTTTCTGAGCTGGATTGG-3′), FH153F (5′-CATGGGCCTAAGCTAAAACG-3′) and FH153R (5′-GTCACATGGGGTTGCTAC-3′). Products were separated by electrophoresis on 4–6% polyacrylamide gels, then images recorded by autoradiography. Allele sizes were estimated by comparison to a sequencing ladder loaded every 24 lanes and African elephant control DNAs were run on every gel. Alleles were designated as their PCR product length.

Genetic analysis

Genetic variation in elephant microsatellite data from 16 loci was analysed within individual loci, as well as used to create composite genotypes for each of the 189 African elephants and the 14 Asian elephants. Only those African elephants that amplified at all 16 loci were included in the subsequent analyses. For each locus, average number of alleles per locus, the average range in allele size and the maximum range in allele size were estimated using MICROSAT (Bowcock *et al*. 1994; Minch *et al*. 1995). Estimates of average observed heterozygosity and associated standard error were performed by ARLEQUIN (version 2.0) (Excoffier *et al*. 1992; Schneider *et al*. 2000). All loci were tested for linkage disequilibrium using ARLEQUIN. Individual genotypes, generated from 16 microsatellite loci, were used to estimate genetic diversity among individuals, populations grouped by site of collection, populations pooled within broad geographical regions and by

classification status as forest or savannah using both MICROSAT and ARLEQUIN.

Genetic distances were estimated using measures based on variance in allele frequencies (VAF) and those based on variance due to repeat number (VRN). As this analysis included populations within two different species of African elephant (Roca *et al*. 2001), the informativeness of both types of measures was compared and contrasted both inter and intraspecies. VAF measures used included proportion of shared alleles (Dps), kinship coefficient (Dkf) and Nei's distance (Gst) as implemented in the MICROSAT program. VRN measures of R_{ST} corrected for unequal and/or small samples sizes (Simon 1997), and $\delta\mu^2$ (delta mu) were computed using MICROSAT.

Phylogenetic trees were constructed using the genetic distances matrix of either Dps (representing a VAF genetic distance) or $\delta\mu^2$ (representing VRN genetic distance) values and clustered by the NEIGHBOR subroutine of the software package PHYLIP (version 3.5) (Felsenstein 1993). Bootstrap iterations (100) were used to estimate the consistency of each node within the derived tree. The programs TREEVIEW (version 1.5) and PHYLODENDRON (version 0.8d) were used to draw the trees (Page 1996; Gilbert 1999).

Population substructure was estimated using analysis of molecular variance (AMOVA) as implemented by the ARLEQUIN program (version 2.0) which partitions the total genetic variation into specified subdivisions and tests for overall levels of differentiation. Both conventional F_{ST} based on allele frequency distributions and R_{ST} corrected by Slatkin (1995) values between all pairs of populations were estimated and tested for significance. Furthermore, populations were tested for deviation from Hardy–Weinberg equilibrium using Fisher's exact test and tested for significance against simulated null distribution as implemented by ARLEQUIN.

The program WHICHRUN, designed to assign unknown individuals to given populations, uses the assumption that the likelihood that an individual comes from a particular population is equal to the Hardy–Weinberg–Castle (H–W–C) frequency of its specific genotype at each locus (Banks & Eichert 2000). Likelihood values for each locus are multiplied to give a series of multilocus likelihood functions represented as logarithm of the odds (LOD) scores for assignment to each of the possible source populations.

Results

Estimates of microsatellite variation measured in 17 African savannah elephant populations (n = 147) and three African forest elephant (including Garamba) populations (n = 42) (Fig. 1) are presented in Table 1. Tests revealed only one locus (FH153) within the forest elephant population Dzanga Sangha not in Hardy–Weinberg equilibrium (data not shown). None of the loci were in linkage disequilibrium. Among 20 collection sites, African elephant heterozygosity was lowest in savannah populations (54–72%) and greatest in the forest elephant populations of Dzanga Sangha (77%), Lope (76%) and Garamba (77%). Among the 17 savannah localities, central Kenya and Namibia showed the lowest average heterozygosities (54%). Overall, average heterozygosity of the three populations of forest elephants was significantly higher than for 17 savannah populations (Mann–Whitney U-test, $P < 0.001$).

Allele size differences consisting of a one base pair (bp) increment in repeat size, due to an insertion/deletion were detected in a total of five loci (FH94, FH48, FH103, FH71 and LAFM03) and occurred in different frequencies across the three species. Asian elephants had a low frequency of these alleles (< 5%) for two loci (FH94 and LAFMS03). Two savannah populations of Namibia in the south and Waza in the north had the alleles with the following frequencies: Waza: FH103 (5%); Namibia: FH103 (18%), LAFMS03 (6%). However, the greatest frequencies were detected in the three forest populations as follows: Garamba: FH48 (5%), FH71 (5%), FH94 (20%), FH103 (30%); Dzanga Sangha FH103 (50%); and Lope FH103 (50%). In population genetic and phylogenetic analyses presented here, these alleles within the five loci were corrected to the next highest repeat class.

Other measures of allelic variation indicated forest populations had nearly double the average number of alleles/locus, and triple the average range in allele size/locus, and an increase by 10 bp for the maximum range in allele size/locus (Table 1A,B). In addition, savannah elephant populations contained few or no alleles that were unique in contrast to the three forest populations (Table 1A). While fewer individuals were tested, forest elephants were consistently higher in measures of diversity. For example, the forest population sample of Lope (n = 7) compared to eight savannah elephant sites of roughly equivalent sample sizes (n = 6–9), was consistently greater in the number of alleles, higher average number of alleles and average allele size range. The most notable difference between forest and savannah elephants was the number of population-specific alleles (Table 1A). Seven savannah populations had no unique alleles and 10 had one or two. By contrast, forest elephants sampled from Dzanga Sangha, Garamba and Lope had 28, 9 and 11 population-specific alleles, respectively.

A relatively broad continuous microsatellite allele distribution in the forest populations is illustrated in Fig. 2 on a per-locus basis. Figure 2 also presents for comparison the allele distribution for the following elephant categories: savannah, forest (without Garamba), Asian and (separately) the Garamba forest elephant population. Most loci show a broad continuous pattern of allele size classes in forest populations (similar in Garamba forest elephants, analysed separately) compared to the

Table 1A Measures of genetic variation across 16 microsatellite loci in African elephants grouped by collection site

Population	No. of individuals	Total no. alleles	Ave. H_O (± SE)	Ave. no. alleles per locus	Ave. range* per locus (bp)	Maximum range* per locus (bp)	No. pop. specific alleles
Savannah							
AB	10	80	0.66 ± 0.16	5.6	11.3	178.0	2
AM	10	72	0.63 ± 0.25	4.5	12.5	178.0	2
BE	6	60	0.64 ± 0.25	3.8	10.7	176.0	0
CH	5	61	0.65 ± 0.30	4.1	11.9	173.8	0
HW	10	70	0.61 ± 0.21	4.5	10.3	177.1	0
KE	9	73	0.54 ± 0.23	4.6	11.25	178.4	1
KR	11	73	0.63 ± 0.26	4.6	12.1	177.3	2
MA	10	63	0.56 ± 0.32	4.2	11.3	179.2	1
MK	2	42	0.72 ± 0.36	2.6	8.1	176.6	0
NA	8	72	0.54 ± 0.25	4.5	11.8	177.6	1
NG	11	74	0.56 ± 0.20	4.6	11.0	176.2	1
SA	9	69	0.56 ± 0.25	4.3	12.0	177.2	1
SE	9	71	0.67 ± 0.20	4.7	11.6	180.0	2
SW	10	74	0.63 ± 0.22	4.6	11.6	178.5	0
TA	9	72	0.63 ± 0.26	4.5	11.0	177.5	0
WA	9	66	0.55 ± 0.21	4.1	11.3	177.6	0
ZZ	9	81	0.69 ± 0.21	5.1	11.9	178.2	1
Forest							
DS	25	175	0.77 ± 0.15	11.0	31.8	186.5	28
GR	10	130	0.77 ± 0.16	8.1	31.8	188.5	9
LO	7	107	0.76 ± 0.26	6.7	27.8	185.0	11

Table 1B Measures of genetic variation across 16 microsatellite loci in different phylogeographical groups of elephants

Phylogeographical group	Locales	No. Ind.	Ave. H_O. (± SE)	No. alleles: Total	No. alleles: Pop. Specific	Ave. no. alleles/locus	Ave. range* (bp)	Max range* (bp)
Asian (14/16 loci)	Asia	14	0.56 ± 0.30	56	15	4.3	9.7	157.6
African	Forest, Savannah	189	0.64 ± 0.14	271	134	16.5	44.8	197.2
Forest	DS, LO, GR	42	0.77 ± 0.11	221	114	13.8	41.0	194.2
Savannah	North-central, Eastern, Southern	147	0.60 ± 0.17	161	30	9.3	21.9	183.2
North-central	BE, WA	15	0.58 ± 0.12	82	1	5.1	13.6	178.6
Eastern	AB, AM, KE, MK, NG, SE, TA	60	0.61 ± 0.17	125	9	8.1	18.0	182.2
Southern	CH, HW, KR, MA, NA, SA, SW, ZZ	72	0.61 ± 0.18	137	10	8.3	18.0	180.8

H_O, average heterozygosity; SE, standard error; bp, base pairs.
*Allele size/locus.
Population abbreviations: AB (Aberdares), AM (Amboseli), BE (Benoue), CH (Chobe), DS (Dzanga Sangha), GR (Garamba), HW (Hwange), KE (Central Kenya), KR (Kruger), LO (Lope), MA (Mashatu), MK (Mount Kenya), NA (Namibia), NG (Ngorongoro), SA (Savuti), SE (Serengeti), SW (Sengwa), TA (Tarangire), WA (Waza), ZZ (Zambezi).

more narrow distribution for nearly all loci among savannah elephant populations.

Significant differences in allele frequency distributions (exact test, $P < 0.01$) were apparent between forest and savannah populations (Fig. 2). One locus was diagnostic (FH127), exhibiting fixed differences in allele size class distributions between savannah (232–290 bp) and forest (150–174 bp). With the exception of two loci (FH67 and FH39), forest elephants had a greater number of alleles (broader range of sizes) at each locus compared with those from savannah. Allele sizes in Garamba elephants generally fell within the combined range observed for other forest elephants for most loci. One exception was locus FH71, which in Garamba exhibited a unique bimodal distribution that was separated by several missing allele size classes. Additionally, a private allele specific to Garamba occurred in locus FH102, and was an outlier relative to allele size range observed in savannah or in other forest populations.

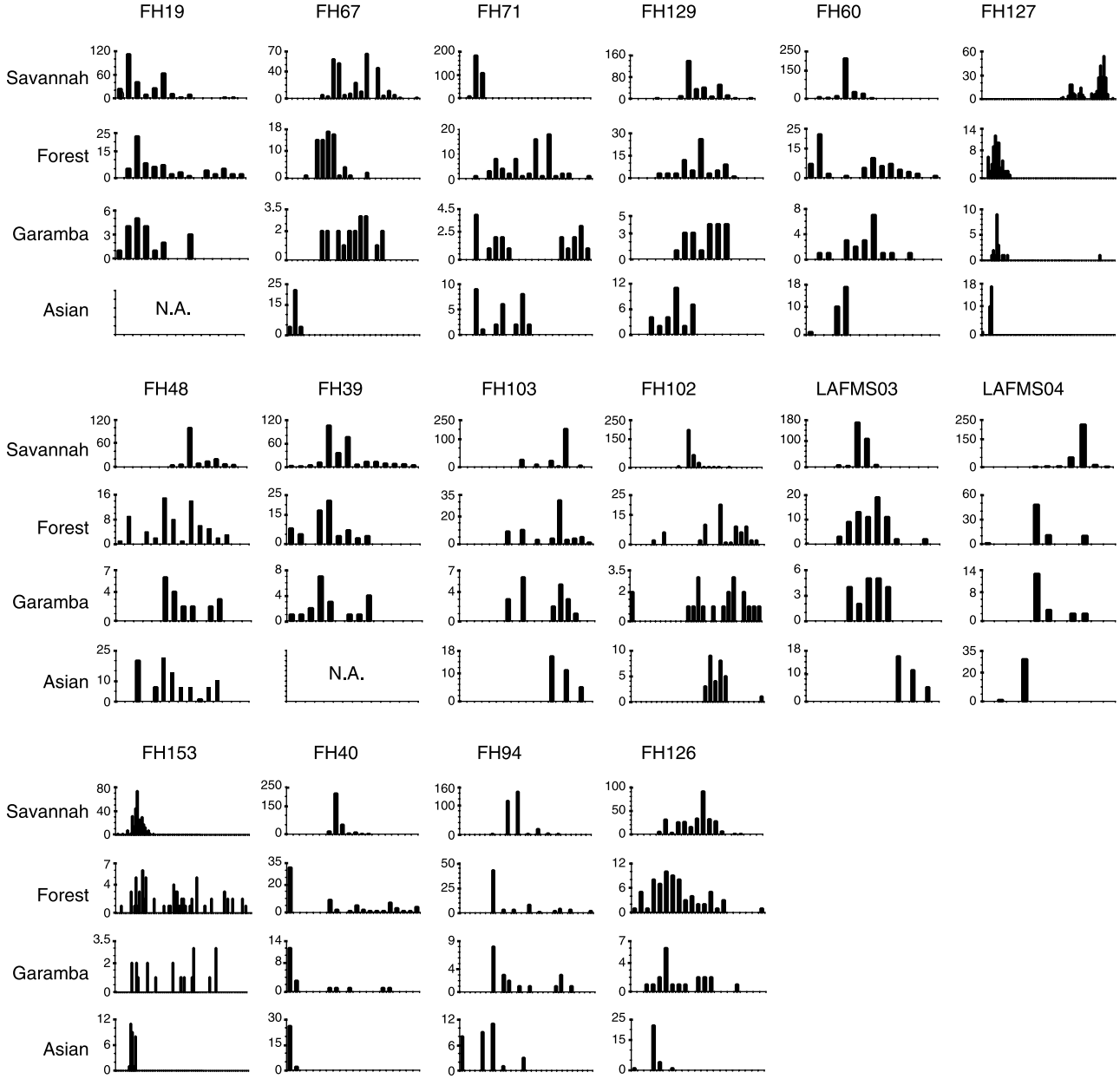

Fig. 2 Frequency distribution of alleles at 16 microsatellite loci among elephants assigned as forest (Dzanga Sangha, Lope), savannah, forest (Garamba) and Asian. The horizontal axes indicate the allele size classes and the vertical axes indicate the number of alleles.

We tested the consistency and robustness of dinucleotide repeat microsatellite loci as an evolutionary marker for elephant speciation. Phylogenetic analysis of composite genotypes for 189 African elephants (Fig. 3) and 14 Asian elephants used Dps and Dkf (VAF) and $\delta\mu^2$ (VRN). Both measures indicated several groupings using individual genotypes (Fig. 3A,B) and population-based analyses (Fig. 3C,D). First, Asian elephants form a monophyletic cluster separate from African elephants. Secondly, African elephants appear to be partitioned into two principal groups consisting of those classified as forest (including Garamba) and those classified as savannah. Almost all Garamba elephant genotypes were interspersed among forest elephants from Lope and Dzanga Sangha (Fig. 3A). Although the same general tree topology is recapitulated in both Dps and $\delta\mu^2$ analyses, branch lengths are markedly different. Disjunct allele distributions between savannah and forest elephants are emphasized by $\delta\mu^2$ because this measure squares the differences in allele sizes in calculating genetic distances.

Among African phylogeographic groupings (Table 1B), elephants from the north-central savannah were the least

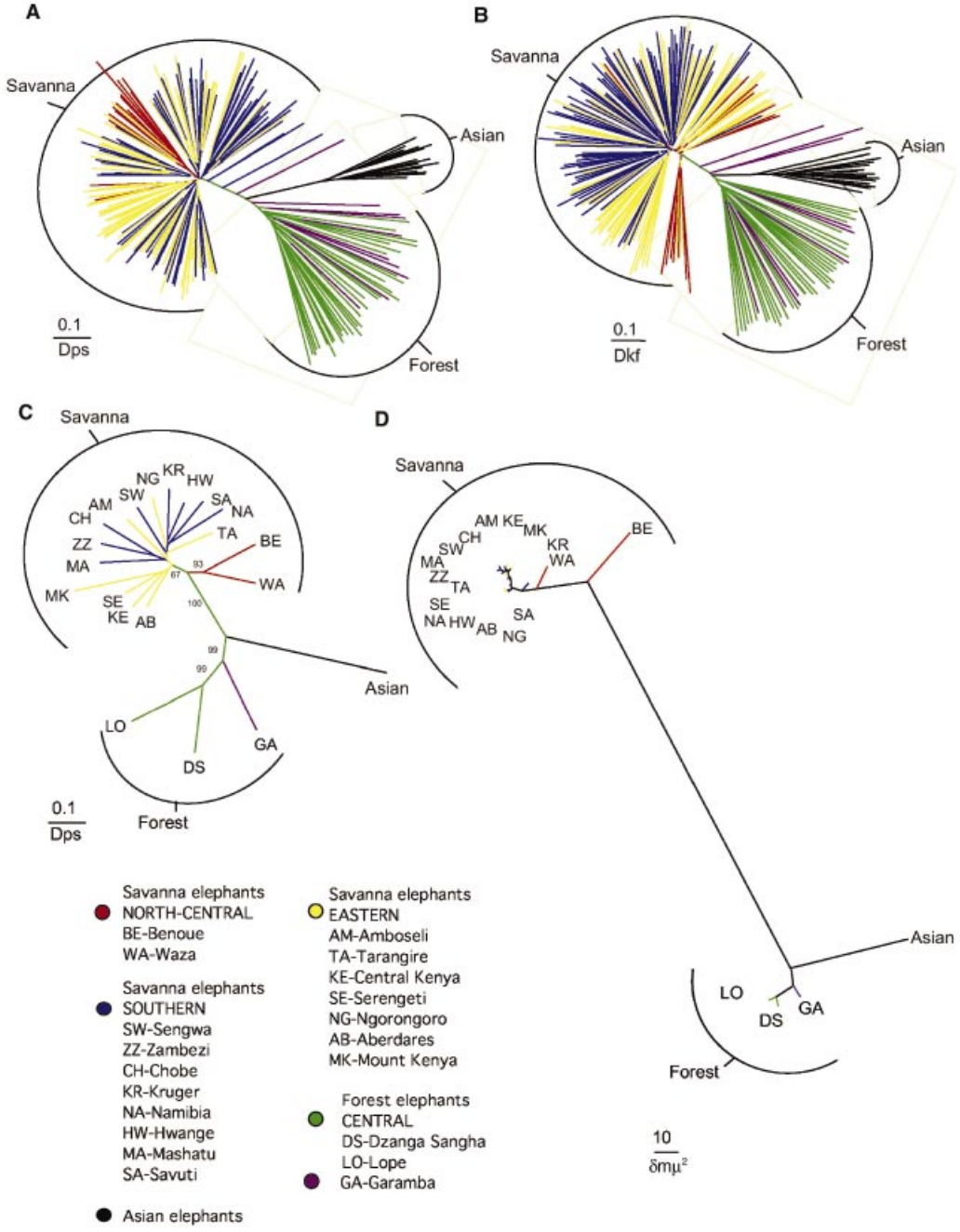

Fig. 3 Phylogenetic relationships among 189 African elephants and 14 Asian elephants. Individuals and groups are colour-coded and/or given a two-letter code as indicated. Bootstrap support (100 iterations) is listed on branches. (A) Neighbour-joining trees based on Dps among individual elephants. (B) Neighbour-joining trees based on Dkf among individual elephants. (C) Neighbour-joining trees based on Dps among elephants grouped into 20 collection sites. (D) Neighbour-joining trees based on $\delta\mu^2$ among elephants grouped into 20 collection sites.

polymorphic with the lowest total number of alleles, average number of alleles and average allele range, although the smaller sample size may, in part, be responsible. The Asian elephants ($n = 14$ individuals) showed lower diversity in all estimates than African elephants ($n = 189$ elephants combining savannah and forest species) (Table 1B). This lower level in Asian elephants could reflect a sampling error from the low number of individuals, an ascertainment bias (Ellegren *et al*. 1995) from developing the microsatellite markers in African elephants (Nyakaana & Arctander 1998; Comstock *et al*. 2000), or both. Among African elephants, populations pooled in accordance with savannah ($n = 147$) or forest ($n = 42$) categories clearly depict greater diversity within forest elephants. Despite having a sample

Table 2 Population pairwise F_{ST} and R_{ST}, and genetic distances between phylogeographical groups of elephants based on composite microsatellite allele frequency distribution

Subdivision	Groups compared	F_{ST}*	R_{ST}	Dps	Dkf	Gst	$\delta\mu^2$	R_{ST}(s)
2 groups			0.614 (total intergroup¶)					
	Asian vs. African#	0.283	0.614	0.824	0.933	0.807	856	0.439
3 groups			0.898 (total intergroup¶)					
	Asian vs. forest†	0.230	0.457	0.756	0.900	0.648	53	0.639
	Asian vs. savannah‡	0.366	0.959	0.874	0.943	0.846	1168	0.578
	Forest vs. savannah‡	0.243	0.896	0.697	0.921	0.698	1245	0.358
4 groups			0.880 (total intergroup¶)					
	Asian vs. forest†	0.230	0.457	0.756	0.900	0.648	53	0.639
	Asian vs. north-central	0.339	0.965	0.864	0.935	0.797	205	0.765
	Forest† vs. north-central	0.229	0.747	0.705	0.914	0.659	180	0.536
	Asian vs. southern + eastern§	0.298	0.962	0.876	0.944	0.854	1276	0.547
	Forest† vs. southern + eastern§	0.225	0.895	0.704	0.921	0.705	1359	0.352
	North-central vs. southern + eastern§	0.070	0.255	0.336	0.673	0.342	1165	0.234
5 groups			0.840 (total intergroup¶)					
	Asian vs. forest†	0.230	0.457	0.756	0.900	0.648	53	0.639
	Asian vs. north-central	0.339	0.965	0.864	0.935	0.797	205	0.765
	Forest† vs. north-central	0.229	0.747	0.705	0.914	0.659	180	0.536
	Asian vs. southern	0.377	0.958	0.876	0.942	0.825	853	0.829
	Asian vs. eastern	0.379	0.971	0.883	0.948	0.879	1683	0.811
	Forest† vs. southern	0.242	0.859	0.708	0.919	0.677	905	0.463
	Forest† vs. eastern	0.237	0.861	0.727	0.925	0.732	1791	0.479
	North-central vs. southern	0.079	0.211	0.368	0.675	0.277	707	0.216
	North-central vs. eastern	0.068	0.331	0.327	0.669	0.404	1597	0.333
	Southern vs. eastern	0.016	0.025	0.199	0.651	0.062	194	0.046

For African elephants, 16 loci were used; 14/16 amplified for Asian elephants.
*$P \leq 0.05$ for each R_{ST} and F_{ST} .
Reynold's (1983) linearized F_{ST} transformed by method of Simon (1997).
#None of the loci are in Hardy–Weinberg equilibrium (HWE) for African elephants in this comparison.
†One locus not in HWE across all forest elephants.
‡Two loci not in HWE across all savanna elephants.
§One locus not in HWE for southern + eastern savanna elephants.
¶Total intergroup variation corresponds to average between-population variance computed by AMOVA (Schneider *et al*. 2000).

size approximately one-third that for savannah elephants, each microsatellite diversity statistic was markedly higher in forest elephants.

To evaluate the extent of population differentiation in elephants, we tested four possible geographical partitions of elephants suggested by Fig. 3, with an analysis of molecular variance (AMOVA) as well as by six different genetic distance measures. In AMOVA, the four partitions were evaluated quantitatively using an R_{ST} estimate of the fraction of variation that can be attributed to geographical subdivision. The results of these calculations are presented in Table 2.

The analysis indicates that 61% of the microsatellite variation (R_{ST}, Table 2) could be explained by differences between Asian vs. African genera, estimated to have diverged about 5 million years ago (Maglio 1973), while 39% of the variation measured with these markers was retained within the continental populations. The amount of variation among African populations accounted for by differences between forest and savannah populations is 90%, with 10% deriving from within the two African species. The total proportion of microsatellite variation accounted for by all intergroup differences (Table 2) was increased to 90% from 61% by subdividing elephants into three groups (Asian, forest, savannah) rather than two (Asian vs. African). However, increasing the number of subdivisions to four by separating north-central from other savannah elephants leads to a reduction (to 88%) in the total proportion of variation accounted for by intergroup differences. This is reduced further (to 84%) when the number of subdivisions is increased to five by including separate north-central, eastern, and southern savannah categories.

With the exception of $\delta\mu^2$, the remaining six genetic distances measures were consistent in depicting species-level

Table 3 Numbers of individuals with log of the odds ratios (LOD) calculated by jackknife iterations to evaluate the chances for correct allocation of these individuals to their sampling groups using the program WHICHRUN. Multilocus likelihood functions were arranged to form ratios of the sampling group with the second most likely allocation group. Fourteen of the 16 loci were used because the loci FH39 and FH19 do not amplify in Asian elephants and WHICHRUN can give erroneous assignment if baseline populations have unequal numbers of loci

Sampling group	% correct assignment*	LOD scores				
		< 0	< 0 × ≤ 5	< 5 × ≤ 10	< 10 × ≤ 20	< 20 × ≤ 30
Forest	95	2	8	18	14	0
North-central	93	1	8	6	0	0
Eastern	62	23	37	0	0	0
Southern	64	26	46	0	0	0
Savannah**	100	0	0	0	96	51
Asian	100	0	0	0	1	13

*Correct assignment is defined as the percentage of individuals assigned to their sampling group with a LOD score greater than 0.
**Savannah group includes all individuals from the North-Central, Eastern and Southern groups.

differences among Asian, forest and savannah elephants. VAF measures consistently indicated the greatest genetic differences were between Asian and savannah elephants, but the VRN measure of R_{ST}(S) suggested the Asian–forest elephants to be more distant. Further subdivision into four and five groups indicated the savannah east and south populations to be more derived. For example, in the five groups analysis, 5/6 measures consistently ranked Asian vs. savannah east and south populations as the more divergent. A comparison of forest and savannah species suggests that forest populations were slightly more closely related to north-central savannah elephants (5/6 measures) relative to either savannah east or south. Overall, $\delta\mu^2$ appeared to be unreliable in elephant microsatellite analysis of phylogeographical relationships. Each category of subdivision was inconsistent with relative ranks of genetic distances, and was not interpretable even with respect to elephant speciation (see three groups).

The phylogeographical subdivision, albeit modest within savannah elephants, may be of use in identifying the broad geographical origins of elephant biological specimens, notably ivory or other products regulated by CITES. To assess the power of population level discrimination, we tested the discriminatory power of available population assignment algorithms that compute a statistical maximum likelihood estimate of population affiliation by assessing an individual genotype's likelihood to be from population A vs. B vs. C., etc., based on microsatellite allele frequencies for each population. The results of this analysis are presented in Table 3. The elephant populations were divided into five groups: Asian, forest (including Garamba), north-central, eastern and southern savannah. The ratio of multilocus likelihood functions was determined for the actual sampling group vs. the most likely allocation group among the other four groups, to determine for each elephant if it would be assigned to its natal population/species. Using this method, all the Asian elephants were assigned correctly with very high LOD scores (Table 3). Similarly, 95% of the African forest elephants were assigned correctly as forest elephant species (the only exceptions being Garamba individuals of established or possible hybrid origin), and 100% of the savannah elephants were assigned to a savannah group. This confirms the large genetic distinctiveness of forest elephants compared to savannah elephants (as was also demonstrated here by the phylogenetic structure and by measures of population subdivision). Assignment of the savannah elephants to their correct geographical subregion was high for the north-central populations, correctly assigned 93% of the time. Among eastern and southern savannah elephants, however, only 64% and 63% of the individuals were assigned correctly to their proper geographical subgroup.

Discussion

Marked genetic divergence of composite microsatellite genotypes is apparent between African forest and savannah elephants. These results show substantial genetic distinctiveness consistent with their recognition as separate species within *Loxodonta*. These data add to the multiple differences in morphology seen between forest and savannah elephants (Groves & Grubb 2000; Grubb *et al*. 2000) and molecular differences in mitochondrial and nuclear genes (Barriel *et al*. 1999; Roca *et al*. 2001). The microsatellite genetic distances computed between forest and savannah populations are almost as great as the difference between Asian and African genera (Table 2), adding further evidence supporting species level distinctions for the two African groups.

The utility of microsatellite loci in phylogeographic and evolutionary analyses of three elephant species varies with methods used to estimate genetic divergence. With the 16 di-nucleotide repeat loci employed here, the

distribution of allele size classes (Fig. 2) varies markedly between forest, savannah and Asian elephants and influenced the performance of different microsatellite genetic distance estimators. Those that were judged most robust (F_{ST}, Dps, Gst and R_{ST}) were consistently informative in analyses of three, four and five phylogeographic groups (Table 2). Both F_{ST}, Dps and Gst are based on variance in allele frequency while R_{ST} utilizes variance in allele size (repeat number). Although informative in pairwise analysis of individuals (Fig. 3B) and populations (Fig. 3D), the usefulness of $\delta\mu^2$ as an indicator of broad phylogeographic divisions depicting a hierarchy of populations within regions within species was minimal. Thus, $\delta\mu^2$ may be the most sensitive to missing allele size classes and/or disjunct distributions that may occur in deeply divergent taxa. Lastly, none of the microsatellite genetic distance measures were linear over the divergence times represented by Asian, forest and savannah elephants. Assuming a constant rate of mutation, then presumably the genetic distance between forest and savannah elephants (2–3 Mya) should be roughly 50–60% that between Asian and African elephants (5 Mya) (Roca *et al*. 2001). However, only one of the six distance measures, R_{ST}(S), was roughly linear over this evolutionary interval (Table 2: 3 groups). Distance measures based on microsatellite loci that consist of tri-, tetra- or more complex repeats may prove to be more linear over this evolutionary interval in elephants.

Across the African continent, savannah and forest (including Garamba) elephants exhibit moderate to high levels of genetic diversity. However, savannah elephant populations have a lower level of genetic diversity by all measures compared to the forest elephant (including Garamba) populations. Considered together, the relative differences in genetic diversity between forest, savannah and Asian elephants provide additional support for the hypothesis that savannah elephants evolved through either a bottleneck or founder event (Roca *et al*. 2001). In addition, the high level of diversity observed in the forest elephant populations may actually be an underestimate since the microsatellite loci were chosen based upon their level of polymorphism in savannah elephants (Nyakaana & Arctander 1998; Comstock *et al*. 2000). The ascertainment bias (Ellegren *et al*. 1995) would favour the observation of higher levels of diversity in savannah elephants relative to forest elephants. The phylogenetic trees and R_{ST} estimates suggest that there has been considerable gene flow between the savannah populations in the eastern and southern regions of Africa at least until fairly recently. This is consistent with field observations (Leuthold & Sale 1973; Georgiadis *et al*. 1994). There appears to have been less gene flow between the north-central populations and those in the east or south.

The forest elephant populations of Dzanga Sangha, Lope and Garamba have high levels of diversity in microsatellite loci. Moreover, these three populations had the greatest incidence and frequency of genotypes possessing an insertion/deletion within five of the 16 microsatellite loci. These allelic variants within locus FH103 occurred at 50% within Dzanga Sangha and Lope, and 30% within Garamba. Four of the five loci with these variant alleles [FH48 (5%), FH71 (5%), FH94 (20%) and FH103 (30%)] all occurred within Garamba. No clear structure defining the three populations is apparent from the phylogenetic analysis of individuals, indicating that sufficient gene flow has occurred among forest elephants from these three collection sites. Phylogenetic trees based upon nuclear introns (Roca *et al*. 2001) had shown Garamba elephants interspersed with forest elephants except for one individual (GR0021) that clustered within the savannah lineage. Similarly, using microsatellite loci, most Garamba elephants were found to possess forest-specific genotypes, including the variant alleles present within loci FH48 and FH71, while a few were intermediate between savannah and forest genotypes (Figs 2 and 3A,B), indicating a history of limited interbreeding in Garamba. Despite this local hybridization in Garamba, the genetic integrity of the two parent species has remained intact (Roca *et al*. 2001), and recognition of species-level distinctions between forest and savannah elephants would therefore not be precluded (Mayr 1969; Barton & Hewitt 1989; O'Brien & Mayr 1991).

The genetic distinction between forest and savannah elephants shown here would lend support to the recognition of two separate species of elephant in Africa. Reclassification will ultimately influence conservation and wildlife management decisions to better accommodate the unique pressures facing forest and savannah elephants. Forest elephants, in particular, may require additional protection as previously impenetrable forests are being opened up by logging and road-building (Tangley 1997). Regardless of species, the rapid loss of habitat and further reduction in numbers due to poaching constitute an immediate threat to all African elephants.

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